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(54) **Method of measuring human c-peptide.**

(57) Human C-peptide in a sample is measured by a sandwich method wherein (a) the sample is contacted with a first antibody specifically recognizing human C-peptide and a second antibody specifically recognizing human C-peptide at a site thereof different from the site recognized by the first antibody, (b) the thus-produced immunoreaction product is separated from the unreacted antibodies, and (c) the immunoreaction product or the unreacted antibodies are determined; or by a competitive method wherein (a) the sample is contacted with a monoclonal antibody specifically recognizing human C-peptide and a labelled human C-peptide, (b) the thus-produced immunoreaction products are separated from the unreacted labelled human C-peptide, and (c) the immunoreaction products or the unreacted labelled human C-peptide is determined.

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This invention relates to a method of measuring human C-peptide by utilizing antibodies specifically recognizing human C-peptide.

Human C-peptide is a peptide composed of 31 amino acid units, which is produced by degradation of proinsulin during the process of biosynthesis of insulin. Human C-peptide is present in an amount equimolar to insulin in pancreatic β-cells, and is secreted into blood according to a stimulus. Accordingly, there is an interrelation between the secretion of active states of insulin and C-peptide. Therefore, by measuring human C-peptide in blood or urine, insulin-dependent diabetes mellitus, insulin-independent diabetes mellitus, insulinoma and insulin autoimmune diseases can be diagnosed. Furthermore, by measuring insulin in blood, the reliability of the diagnosis can be enhanced.

As the methods of measuring C-peptide, there are known the radioimmunoassay utilizing a competitive method using polyclonal antibodies (see Japanese Unexamined Patent Publications No. 52-25767, No. 57-37586 and No. 62-132172), and the enzyme imunoassay utilizing a competitive method using polyclonal antibodies (see Japanese Unexamined Patent Publication No. 1- 165962).

In the known radioimmunoassay, since a radioactive isotope is used, problems arise with respect to the safety of an operator and disposal of the isotope and other material, and arise further in that a special equipment and a special measuring device are required with regard to the use of the radioactive substance.

Furthermore, the use of polyclonal antibodies has problems in that it is not easy to prepare serums having constant and uniform performances and the maintenane of quality of the polyclonal antibodies is troublesome. The technical problem underlying the present invention is to solve the foregoing problems involved in the conventional techniques and provide a method of measuring human C-peptide whereby human C-peptide can be measured with a high precision by a simple operation. The solution to this technical problem is achieved by providing the embodiments characterized in the claims.

Thus, in one aspect of the present invention, there is provided a method of measuring human C-peptide which comprises the steps of (a) contacing a sample containing human C-peptide with a first antibody specifically recognizing human C-peptide and a second antibody specifically recognizing human C-peptide at a site thereof different from the site recognized by the first antibody, (b) separating the thus-produced immunoreaction product from the unreacted antibodies, and (c) determining the immunoreaction product or the unreacted antibodies.

In another aspect of the present invention, there is provided a method of measuring human C-peptide which comprises the steps of (a) contacting a sample containing human C-peptide with a monoclonal antibody specifically recognizing human C-peptide and a labelled human C-peptide, (b) separating the thus-produced immunoreaction products from the unreacted labelled human C-peptide, and (c) determining the immunoreaction products or the unreacted labelled human C-peptide.

The present invention will now be described in detail.

The first method of the present invention, i.e., the sandwich method, is first described. In this method, monoclonal antibodies or polyclonal antibodies can be used for the immunoassay. The monoclonal antibodies as used can be prepared by a known process (see,for example, G. Kohler, C. Milstein et al; Nature, 256, 495, 1975). The monoclonal antibodies used in the examples given hereinafter are prepared according to this process. The polyclonal antibodies as used can be an antiserum to human C-peptide or a mixture of monoclonal antibodies.

Preferably, the first antibody specifically recognizing human C-peptide is fixed to a solid phase to facilitate the separation of the immunoreaction product from the unreacted antibodies after the immunoreaction. A known method can be adopted for fixing the first antibody to a solid phase. As the solid phase, there can be mentioned, for example, polystyrene, polyethylene, polyvinyl chloride, polycarbonate, cephalose particles, latexes, agarose, cellulose and polymethacrylates.

Preferably, the second antibody specifically recognizing human C-peptide is labelled to facilitate detection of the immunoreaction product or the unreacted second antibody after the immunoreaction. The method of labelling the second antibody and the method of detecting the immunoreaction product or the unreacted second antibody are not particularly limited, and the labelling and detection can be conducted by known methods. The labelling substance that can be used in the present invention is not particularly limited. As the labelling substance, there can be mentioned, for example, enzymes such as peroxidase, β-D-galactosidase, alkaline phosphatase, urease, catalase and β-glucronidase; radioactive substances such as ^3H, ^{125}H and ^{131}I; and fluorescent substances such as fluorescamine, fluorescein isothiocyanate and tetrarhodamine isothiocyanate (TRITC).

When the second antibody is not labelled, it is preferable to use a labelled third antibody specifically recognizing the immunoreaction product or the second antibody to facilitate the detection of the immunoreaction product or the unreacted antibodies.

When the sandwich method is carried out by using the above-mentioned reagents, the order of the contact of the reagents with the sample is not particularly limited. The sequential contact method can be adopted wherein the reagents are sequentially contacted with the sample, or the immunoreaction can be performed by incorporating together the reagents at a time with the sample. When the second antibody is not labelled and the labelled third antibody is used, the labelled third antibody may be incorporated either in a reaction mixture of the sample, the first antibody and the second antibody in the step (a), or, in the immunoreaction product or the unreacted antibodies after the immunoreaction product is separated from the unreacted antibodies in the step (b). Finally, the immunoreaction product, the unreacted second antibody or the labelled third antibody is detected.

The second method of the present invention, i.e., the competitive method, will now be described. A monoclonal antibody is used in the competitive method, and the monoclonal antibody used is preferably fixed to a solid phase. Furthermore, labelled human C-peptide is used in the competitive method, and the labelled human C-peptide and human C-peptide in a sample are competitively reacted with the monoclonal antibody. The labelling substance used and the labelling manner can be the same as those described in the above-mentioned sandwich method. The immunoreaction products are separated from the unreacted labelled human C-peptide, and either the label of the unreacted labelled human C-peptide or the label of the immunoreaction product produced from the labelled human C-peptide is detected. The monoclonal antibody is preferably fixed to a solid phase, and the solid phase used and the manner of using the solid phase in the competitive method can be the same as those described in the above-mentioned sandwich method.

In addition to the above-mentioned substances, conventional reagents such as a substrate, a buffer agent, a rinsing agent and a reaction stopper can be used for the measurement in the sandwich method and the competitive method.

According to the present invention, a plurality of samples can be measured at a high sensitivity and a high safety in a short time by a simple operation as compared with the conventional measuring methods. The measurement of human C-peptide in a sample can be advantageously carried out at a concentration of 0.1 to 50 ng/ml.

The present invention will now be described in detail with reference to the following examples that by no means limit the scope of the invention.

Example 1

Measurement of Human C-Peptide with Use of Two Kinds of Monoclonal Antibodies

(A) Fixation of anti-human C-peptide antibody

In each well of an untreated microtiter plate (96 wells, Nunk plate supplied by Intermed Co.) was charged with 100 μl of a solution prepared by dissolving 10 μg/ml of mouse-derived anti-human C-peptide antibody (hereinafter referred to as "first antibody") in 0.1M sodium carbonate buffer having a pH value of 9.6, and incubation was carried out at 4°C overnight.

Then the solution was removed from each well and the plate was rinsed three times with a solution (hereinafter referred to as "PBS-T") prepared by dissolving 0.04% by weight of Tween-20 (surfactant) in a phosphoric acid-buffered physiological saline solution having a pH value of 7.2 (0.01% by weight of phosphoric acid buffer containing 0.85% by weight of NaCl; hereinafter referred to as "PBS"). Then 300 μl of PBS-T solution containing 1.0% by weight of bovine serum albumin (hereinafter referred to as "BSA") was charged in each well, and the blocking treatment was carried out at 4°C and the anti-human C-peptide antibody-fixed microtiter plate was stored in this state.

(B) Preparation of antibody labelled with alkaline phosphatase (hereinafter referred to as "AP")

A solution of N-succinimidyl-3-(2'-pyridyldithio)-propionate in ethanol was added to a solution of AP (5 mg/ml) in PBS, the mixture was maintained at room temperature for one hour to effect a reaction, and the reaction mixture was dialyzed with PBS.

Separately, a solution of S-acetylmercaptosuccinic anhydride in 1,4-dioxane was added to a solution of 1 mg of a mouse-derived anti-human C-peptide monoclonal antibody (recognizing human C-peptide at a site thereof different from that recognized by the first antibody) in PBS, the mixture was maintained at 30°C for one hour to effect a reaction, and the reaction mixture was dialyzed with PBS.

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Then the AP solution and the solution of the mouse-derived anti-human C-peptide monoclonal antibody were mixed together, a 1M solution of hydroxylamine was added to the mixture, and the resultant mixture was allowed to stand at 4°C overnight. An AP-labelled anti-human C-peptide monoclonal antibody was refined from the thus-obtained reaction solution by the high speed liquid chromatography using TSK-Gel G-3000SW (trademark; supplied by Tosoh Corp.).

(C) Determination of C-peptide in sample

The temperature of the anti-human C-peptide antibody-fixed microtiter plate prepared by the method described in (A) of this example was returned to room temperature and the plate was rinsed with the PBS-T solution, and 50 μl of a standard sample containing human C-peptide at a concentration of 0 to 50 ng/ml was incorporated in each well. The AP-labelled anti-human C-peptide monoclonal antibody prepared by the method described in (B) of this example was diluted with the PBS-T, and 50 μl of the dilution was incorporated in the well. Incubation was carried out in this state at room temperature for 2 hours, and the solution was removed and rinsing with the PBS-T solution was conducted three times. Then 100 μl of a substrate solution composed of 3 mg/ml of p-nitrophenyl phosphate and a 50 mM of a carbonic acid buffer containing 10 mM of magnesium chloride and having a pH value of 9.5 was incorporated in each well. An enzyme reaction is effected at room temperature for 30 minutes and then 100 μl of a 1N sodium hydroxide solution was added to the reaction mixture to stop the enzyme reaction.

The determination of human C-peptide was conducted with respect to each well of the microtiter plate by measuring the absorbance intensity at a wavelength of 405 nm and a reference wavelength of 492 nm by using an automatic titer plate reader (Model MPR-A4 supplied by Tosoh Corp.). The results are shown in Table 1.

TABLE 1

Concentration C-peptide (ng/ml)	of Absorbance intensity
0	0.01
0.1	0.05
0.3	0.24
1.0	0.93
3.0	1.55
10	2.10
30	2.46
50	2.60

Example 2

Measurement of Human C-Peptide Using Polyclonal Antibody and Monoclonal Antibody

The procedures described in Example 1 were repeated wherein a solution of anti-human C-peptide antiserum derived from a rabbit was used as the fixation antibody instead of the anti-human C-peptide antibody used in the method described in (A) of Example 1, with all other conditions remaining substantialy the same. The results of the human C-peptide determination are shown in Table 2.

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TABLE 2

Concentration of C-peptide (ng/ml)	Absorbance intensity
0	0.01
0.1	0.12
0.3	0.34
1.0	0.80
3.0	1.17
10	1.30
30	1.49
50	1.59

Claims

1. A method of measuring human C-peptide which comprises the steps of (a) contacting a sample containing human C-peptide with a first antibody specifically recognizing human C-peptide and a second antibody specifically recognizing human C-peptide at a site thereof different from the site recognized by the first antibody, (b) separating the thus-produced immunoreaction product from the unreacted antibodies, and (c) determining the immunoreaction product or the unreacted antibodies.

2. A method as claimed in claim 1 wherein the first antibody is fixed to a solid phase.

3. A method as claimed in claim 1 or 2 wherein the second antibody is labelled with a labelling substance selected from the group consisting of enzymes, radioactive substances and fluorescent substances.

4. A method as claimed in claim 1 or 2 wherein the second antibody is not labelled; and a labelled third antibody specifically recognizing the second antibody is incorporated either in a reaction mixture of the sample, the first antibody and the second antibody in the step (a), or, in the immunoreaction product or the unreacted antibodies after the immunoreaction product is separated from the unreacted antibodies in the step (b).

5. A method of measuring human C-peptide which comprises the steps of (a) contacting a sample containing human C-peptide with a monoclonal antibody specifically recognizing human C-peptide and a labelled human C-peptide, (b) separating the thus-produced immunoreaction products from the unreacted labelled human C-peptide, and (c) determining the immunoreaction products or the unreacted labelled human C-peptide.

6. A method as claimed in claim 5 wherein the monoclonal antibody is fixed to a solid phase.

7. A method as claimed in claim 5 or 6 wherein the human C-peptide is labelled with a labelling substance selected from the group consisting of enzymes, radioactive substances and fluorescent substances.

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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
Y	EP-A-0 227 351 (SHIONOGI & COMPANY) *See whole document*	1-8	G01N33/74 G01N33/68 G01N33/577 C07K15/00
Y	ACTA ACADEMIAE MEDICINAE SINICAE vol. 11, no. 1, February 1989, CHINA page 51; C. WU ET AL: 'Studies on monoclonal antibodies directed against human c-peptide.' *See whole abstract*	1-8	
A	DIABETES RESEARCH AND CLINICAL PRACTICE vol. SUPPL, no. 1, 1985, WASHINGTON DC USA pages 18 - 19; L. ANGELO ET AL: 'A highly sensitive monoclonal antibody against human c-peptide.' *See whole abstract*	1-8	
A	ENDOCRINOLOGY vol. 113, no. 6, June 1986, WASHINGTON DC USA pages 2135 - 2144; O.D. MADSEN ET AL: 'The production and characterization of monoclonal antibodies specific for human proinsulin using a sensitive microdot assay procedure.' * See whole article*	1-8	**TECHNICAL FIELDS SEARCHED (Int. Cl.5)** G01N C07K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	26 FEBRUARY 1992	VAN BOHEMEN C.G.